The Turnover of the Lipid Components of Myelin

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Abstract

The rate of loss of radioactivity in the lipid components of rat myelin labeled with acetate-1-C^{14} was determined over a period of one year. Rats were injected with acetate-1-C^{14} at 15–16 days of age and purified myelin was prepared by differential ultracentrifugation from brain and spinal cord of this group at 1 day, 2 weeks, 1 month, 2 months, 3 months, 6 months and 1 year after injection. Total lipid was extracted from the myelin preparations and the lipids were separated into their components by thin-layer chromatography. Cholesterol, galactolipid, ethanolamine phosphatide, choline phosphatide, inositol phosphatide, serine phosphatide and sphingomyelin specific activities at each age were measured. Three of the myelin lipid components, serine phosphatide, inositol phosphatide, and choline phosphatide decreased in specific activity faster than cerebroside, cholesterol, sphingomyelin, and ethanolamine phosphatide. Acetate-1-C^{14} injected into adult animals, though incorporated into myelin to a very small extent, is taken up primarly in the choline phosphatide. These experiments suggests that myelin does not behave as a fixed entity but that certain constituents may be more actively metabolized than others.

Introduction

THE RELATIVE METABOLIC STABILITY of the lipids of myelin is well known. In 1959 Davison reported that cholesterol-4-C^{14} incorporated into brain lipids while myelination is proceeding rapidly in young animals was retained for at least 12 months (1). The radioactivity of the white matter showed little indication of turnover during this time. Since then Davison has extended his turnover studies to phospholipids using as markers serine-3-C^{14} (2), P^{32} (3), glycerol-1-C^{14} (4), and to sulfatides using S^{35} (5). All his findings from these studies indicate that lipids once incorporated into myelin are metabolized only very slowly. In more recent work where myelin was isolated and purified, the myelin lipid components were found to turn over at the same slow rate (6). Since the markers used by Davison would not be expected to label extensively the fatty acids of the various lipids, we have re-examined the turnover of myelin lipids in the rat using acetate-1-C^{14} as a marker. In this work we have utilized the newly developed methods for the preparation of purified myelin as well as thin-layer chromatography for the separation of the major myelin lipids. A preliminary account of this work was reported earlier (7).

Methods and Materials

Animals

Wistar rats, both sexes, 15 or 16 days of age were injected with sodium acetate-1-C^{14}, 1 $\mu c/g$ body weight. Groups of these animals were killed at 1 day, 2 weeks, 1 month, 2 months, 3 months, six months, and 1 year after injection, and the brains and spinal cords removed quickly.

Preparation of Subcellular Fractions

Purified myelin was prepared from these animals by the method of Laatsch et al. (8). All sucrose solutions were dissolved in the adenosine triphosphate-phosphate-$MgCl_2$ buffer of Sporn et al. (9). The nonmyelin fraction which precipitated in 0.88 M sucrose was centrifuged at 800 × g, the precipitate resuspended and centrifuged again three times to remove the heavy elements, such as nuclei, blood cells, blood vessels and cell walls. The supernatant fluids free of these materials were pooled and the particulate matter collected in a pellet, resuspended in 0.88 M sucrose layered above with 0.25 M sucrose, recentrifuged at 105,000 × g for one hour to remove myelin contamination. The resulting precipitate, which was rich in succinic dehydrogenase activity, was termed the "mitochondrial rich" fraction. Besides mitochondria this preparation may be expected to contain axoplasmic fragments, synaptic membranes, and nerve ending particles (10).

Succinic Dehydrogenase Assays

Succinic dehydrogenase was assayed in the myelin and "mitochondrial-rich" fraction by the procedure described by Laatsch et al. (8). Protein determinations were made by the method of Lowry et al. (11).

Lipid Extraction

Total lipids of the myelin and mitochondrial-rich fraction were extracted with 20 volumes of chloroform-methanol (2:1 v/v) and filtered through glass wool. The lipid extracts were washed with 0.88% KCl and "upper phase" solutions as outlined by Folch et al. (12). The washed solutions were evaporated to dryness in a rotating evaporator, the dried material redissolved in chloroform-methanol, and filtered through glass wool to remove the denatured protein. The lipid solutions were stored at −20C until analyses were performed.

Thin-layer Chromatographic Separation of Lipids

Approximately one milligram lipid samples were applied to each of two plates and separated by thin-layer chromatography, using the two dimensional method of Skidmore and Entenman (13). The following solvents were used: solvent I: chloroform-methanol-7N NH_4OH 60:35:5 v/v/v/; solvent II: chloroform-methanol-7N NH_4OH 35:60:5 v/v/v/; solvent III: chloroform-methanol-H_2O 65:25:4 v/v/v/ (14).

Migration of solvent I in the vertical dimension and solvent II in the horizontal dimension resulted in good separation of all components except for an ill-defined sulfatide-cerebroside spot. When it was desired to obtain cerebroside and sulfatide well separated from each other, solvent III was used in place of solvent II. This system, however, did not produce a well-defined inositol phosphatide spot. The

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TABLE I

Succinic Dehydrogenase Activity of Fractions

		Myelin	Enriched mitochondria	Mitochondria Myelin
		SDH activity[a]		
Laatsch et al.		0.1	2.5	25.0
9/ 6/63	Adult	0.04	3.93	98.2
10/ 3/63	Adult	0.13	4.01	30.8
	Adult	0.08	3.67	45.9
11/ 5/63	Adult	0.25	10.8	43.2
	Adult	0.35	9.86	28.2
3/ 5/64	Adult	0.38	7.01	18.5
3/12/64	2 Week	0.38	7.76	20.4
4/ 2/64	1 Mo.	0.019	2.64	138.0
4/28/64	2 Mo.	0.014	2.96	211.0
7/24/64	2 Mo.	0.18	4.22	23.6

[a] Moles/kg protein/hour.

corresponding spots from each of the two plates were scraped, pooled, and the lipids eluted by successively shaking the silicic acid scrapings with 2-2 ml portions of solvent I, 2–2 ml portions of solvent II, and 1–2 ml portion of methanol. Each eluate was filtered through glass wool and the eluates from each spot pooled. Half of each sample was evaporated, plated, and counted for radioactivity with a gas-flow counter, the other half used for phosphorus (15), cerebroside (16), or cholesterol (17) assay. Each extract was separated in this way in duplicate. Although certain of the phospholipid compounds are known to be a mixture of plasmogenic and diacyl forms, these were not separated from each other and each class was treated as a single compound. Results are expressed as counts per minute per milligram of lipid. In calculating the results, the molecular weight of the phosphatides and cerebroside was assumed to be 800, the sulfatide, 900.

Results

Criteria of Purity

The ratios of the succinic dehydrogenase activities in the myelin and mitochondrial-rich fractions are shown in Table I. The activities of the enzyme obtained by Laatsch et al. (8) in the guinea pig fractions are compared with those in a sampling of our preparations. In most cases the myelin contained very little succinic dehydrogenase activity. A myelin stain applied to conventional histological

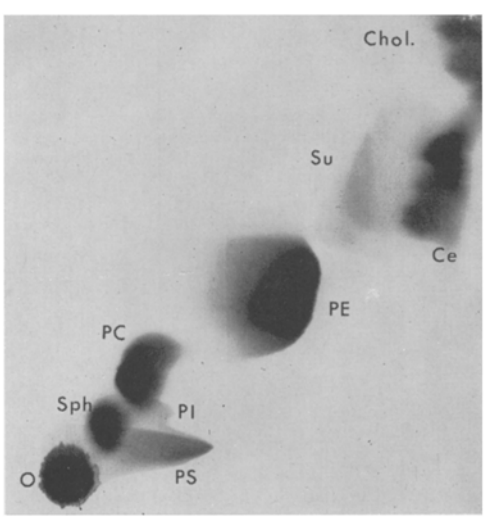

FIG. 1. Thin-layer chromatogram of myelin lipid. Solvent in vertical dimension: chloroform-methanol-7N NH_4OH 60: 35:5 v/v/v: Solvent in horizontal dimension: chloroform-methanol - H_2O 65:25:4 v/v/v. O = origin, PS = serine phosphatide, Sph = spingomyelin, PI = inositol phosphatide, PC = choline phosphatide, PE = ethanolamine phosphatide, Su = sulfatide, Ce = cerebroside, Chol = cholesterol. Spots are visualized in iodine vapor.

TABLE II

Composition of Lipid Extract
% Dry Weight

	Myelin fraction	Mitochondria-rich fraction
% Phospholipid	42.3	78.0
Sphingomyelin	6.1	6.0
Serine phosphatide	6.1	4.7
Inositol phosphatide	1.5	3.6
Choline phosphatide	10.3	29.7
Ethanolamine phosphatide	19.1	25.1
Lysolecithin	.....	3.1
Phosphatidic acid	.....	2.5
Sulfatide	6.7 }	5.2
Cerebroside	24.1 }	
Cholesterol	24.3	15.4
Protein	3.5	3.1
Total %	101.7	98.4

preparations of a portion of the pellets revealed well-stained homogenous granules in the myelin preparations and no uptake of stain in the mitochondrial sections. Electron microscope pictures of the myelin showed typical myelin membranes to be present in our myelin preparations.

Lipid Composition of Fractions

A typical lipid composition expressed in per cent lipid in each fraction (Table II) showed a great difference between the myelin and mitochondrial-rich fractions. The most striking difference is in the phospholipid content, 42.3% in the myelin vs. 78.0% in the mitochondrial-rich fraction. Major differences are also seen in quantities of choline phosphatide and cerebroside plus sulfatide between the two preparations. The lipid composition of the myelin fraction compares closely to the values for per cent phospholipid, cholesterol, and galactolipid obtained by Autilio et al. for ox myelin (18). Although our mitochondrial-rich fractions had a high phospholipid content similar to that reported by Lϕvtrup for brain mitochondria (19), our assays showed far more cholesterol (15.4% vs. 5.0%) and much less galactolipid (5.2% vs. 18.0%). The explanation for these differences is not apparent.

Radioactivity of Total Lipid Fractions

The radioactivity of the acetate-1-C^{14} label at 1 day, 2 weeks, 2 months, and 6 months in myelin and mitochondrial-rich fractions is shown in Table III. Acetate-1-C^{14} is incorporated nearly equally into the two fractions from brain and into those from spinal cord. The label is lost more rapidly from the mitochondrial-rich fractions in spite of the fact that a four- to sixfold dilution due to formation of new myelin occurs in the brain myelin fraction in six months. This dilution is even greater in the spinal cord. We have estimated that a twofold dilution occurs in the brain in the mitochondrial-rich lipid during this time. After six months the specific activity of the brain myelin lipids is about 1/7 of its original activity while the mitochondrial lipids are 1/22 of the activity at one day (uncorrected for dilution). Spinal cord lipids are 1/11 and 1/35 of

TABLE III

Radioactivity of Total Lipid of Fractions

	1 Day cpm/mg	2 Weeks cpm/mg	2 Months cpm/mg	6 Months cpm/mg
Myelin				
Brain	4830	2700	1150	700
Spinal cord	3830	2025	525	335
Mitochondria-rich				
Brain	5080	2180	570	230
Spinal cord	3720	834	194	106

TABLE IV

Radioactivity of Separated Myelin Lipids

	1 Day cpm/mg	2 Weeks cpm/mg	6 Months cpm/mg
Brain myelin	4830	2700	700
Sphingomyelin	2500	1010	490
Cerebroside	6048	1880	800
Ethanolamine phosphatide	3220	1445	500
Cholesterol	5000	2640	640
Serine phosphatide	2040	900	200
Choline phosphatide	4760	1020	200
Inositol phosphatide	2000	360	70
Spinal cord myelin	3830	2025	335
Sphingomyelin	2285	770	240
Cerebroside	3500	1500	282
Ethanolamine phosphatide	3060	931	184
Cholesterol	4540	1035	240
Serine phosphatide	2000	605	100
Choline phosphatide	3260	750	92
Inositol phosphatide	1400	266	.....

the original activity of the myelin and mitochondrial-rich fractions respectively. These figures support the findings of Davison, that myelin lipid has a relatively slow rate of turnover.

Separation of Lipids

The lipids were separated into their component parts by thin layer chromatography. Figure 1 shows a typical lipid separation using solvents I and III. Sphingomyelin, serine phosphatide, inositol phosphatide, (probably the monophosphoinositide), choline phosphatide, ethanolamine phosphatide (containing large amounts of plasmalogens, sulfatide, cerebroside, and cholesterol appear well separated from each other. Sulfatide and cerebroside showed no difference in rate of turnover, therefore these were treated as a unit. The specific activities of the brain and spinal cord myelin lipid components at 1 day, 2 weeks, and 6 months are listed in Table IV. The greatest uptake in radioactivity at one day after injection of acetate-1-C^{14} was in cerebroside,

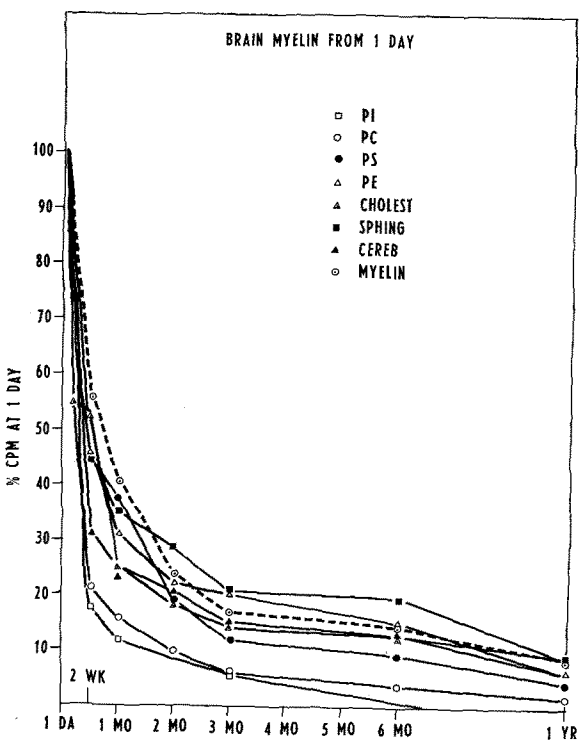

FIG. 2. Specific activities of brain myelin lipids expressed as per cent counts per minute at 1 day after injection. PI = inositol phosphatide, PC = choline phosphatide, PS = serine phosphatide, PE = ethanolamine phosphatide, cerebroside includes sulfatide.

TABLE V

Uptake of Acetate-1-C^{14} into Lipid Fractions of Adult Rats

	Total lipid cpm/mg	Sphing. cpm/mg	PS cpm/mg	Lec. cpm/mg	PE cpm/mg	Cereb. cpm/mg	Cholest. cpm/mg
Brain myelin							
2 hour	35	19	20	74	22	3	24
24 hour	56	22	31	117	32	13	33
Brain mitochondria							
2 hour	238	60	40	285	100	36	105
24 hour	264	45	80	390	125	50	110

cholesterol, and choline phosphatide fractions. At the end of six months the cerebroside had retained four times as much activity as the choline phosphatide. The relationships for brain myelin are shown graphically in Figure 2 where the specific activity of each lipid at each age is expressed as the per cent of the counts per minute per milligram on day 1 after injection. Each point represents the average of 3–6 different groups of animals. The greatest loss of specific activity occurs in the first month after injection, when great dilution due to formation of new myelin occurs. Inositol phosphatide and choline phosphatide show a greater loss of specific activity than other myelin lipids. At 6 months after injection the choline phosphatide specific activity is 4% of the original, inositol phosphatide about 1%, and serine phosphatide about 10%. The other myelin lipids including cholesterol range from 13–20% of their original activity. When the losses of radioactivity of the myelin components are recalculated utilizing the specific activity at two weeks as 100%, the initial period of great dilution is avoided (Fig. 3). Again, at 6 months the inositide is shown to lose its activity most rapidly followed by choline phosphatide and serine phosphatide. Other myelin lipids show slower rates of turnover with cholesterol taking an intermediate position. Approximately the same relationships are seen in spinal cord myelin except that the radioactivity in inositol phos-

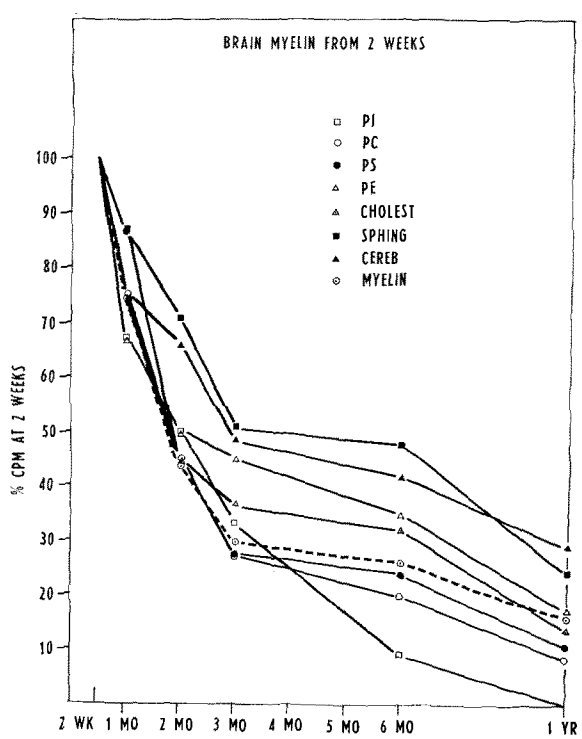

FIG. 3. Specific activities of brain myelin lipid expressed as per cent counts per minute at 2 weeks after injection.

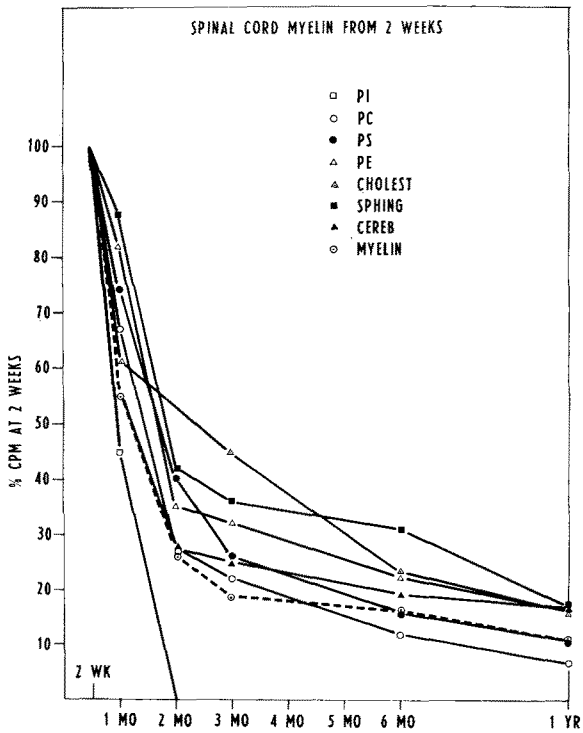

FIG. 4. Specific activities of spinal cord myelin lipids expressed as per cent counts per minute at 2 weeks after injection.

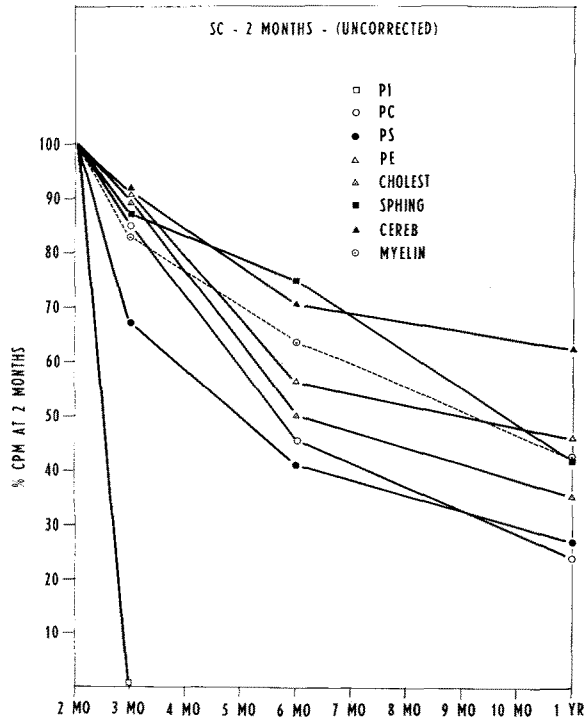

FIG. 5. Specific activities of spinal cord myelin lipids expressed as per cent counts per minute at 2 months after injection.

phatide completely disappears early (Fig. 4).

Figure 5 shows turnover rates of lipid components of adult spinal cord myelin using specific activities at 2 months as the 100% levels. Again, differences in rates of turnover are seen in the lipids, with choline phosphatide and serine phosphatide (after inositol phosphatide) losing their activities more rapidly than the other lipids.

We have determined that some dilution of the myelin occurs after 2 months. By measurement of cerebroside quantities and myelin yields at each age we have estimated correction factors for dilution after 2 months. This estimation is more difficult at earlier ages when rapid myelin synthesis is proceeding. In Figure 6 these factors were applied and corrected turnover rates without dilution are recorded. We have tentatively assigned half-life numbers for each lipid in adult myelin. Inositol phosphatide, choline phosphatide and serine phosphatide, the relatively active components show half-lives of 5 weeks, 2 months, and four months, respectively. Ethanolamine phosphatide and cholesterol show comparable half-lives at 7–8 months while sphingomyelin and galactolipid turn over more slowly with estimated half-lives of 10 months and 14 months. The latter value for galactolipid is somewhat longer than the 280-day value assigned by Davison (5) to sulfatide. We have eliminated the early period of more rapid metabolism, however, by utilizing the values at 2 months as the 100% level.

These relationships may be compared with similar data for lipids from the mitochondrial-rich fraction. The rate of loss of specific activity of the lipids corrected for dilution are seen in Figure 7. Again, inositol phosphatide, choline chosphatide, and serine phosphatide show the highest rate of turnover with ethanolamine phosphatide and sphingomyelin following. In the mitochondrial fraction cholesterol appeared to be the longest lived component. Values are not shown for cerebroside, but it behaved much like cholesterol.

Short-term Uptake of Acetate-1-C^{14} into Adult Myelin

Acetate-1-C^{14}, 250 μc/rat was injected into each of four 250 g. rats 6 months of age. Myelin and mitochondrial-rich fractions were prepared at 2 hr and 24 hr after injection. The specific activities of the separated lipids are shown in Table V. Comparatively little label was incorporated into the myelin lipids, especially in 2 hr. Choline phosphatide shows far more incorporation in both myelin and mitochondrial-rich fractions at 2 hr and 24 hr. These results support our findings in the long-term experiments, that choline phosphatide is one of the more metabolically active lipids in brain. The inositol phosphatide was lost in this experiment and serine phosphatide showed little uptake of the acetate-1-C^{14}.

Discussion

The data shown indicate somewhat different rates of turnover for various lipids of myelin labeled with acetate-1-C^{14}. In choline phosphatide we have found the carbon label predominantly in the fatty acids. We assume this to be the case in the other lipids also. Davison found that myelin lipids labeled with P^{32}, or glycerol-1-C^{14} appear to turn over together and at the same slow rate (6), the myelin, therefore, acting as a metabolic unit. These labels, however would not be expected to label the fatty acids significantly. In our experiments inositol phosphatide, choline phosphatide, and serine phosphatide appear to be most rapidly metabolized. Ansell and Dohmen, using P^{32}, found inositol phosphatide to have the highest rate of synthesis of the phospholipids in brain with choline phosphatide following (20). Serine phosphatide, on the other hand, incorporated P^{32} very slowly. In our experiments acetate-1-C^{14} incorporated into serine

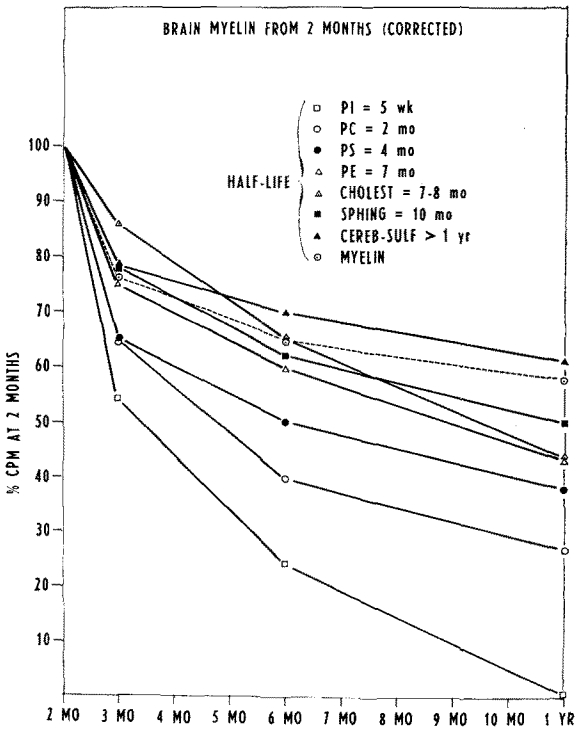

FIG. 6. Specific activities of brain myelin lipids expressed as per cent counts per minute at 2 months after injection. These values are corrected for dilution.

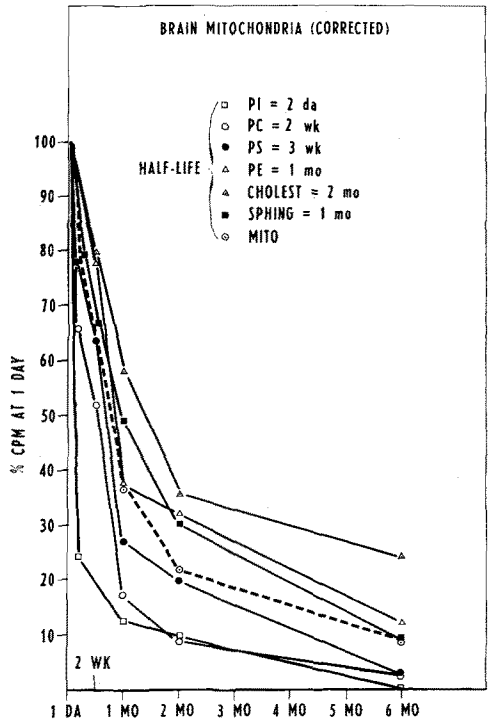

FIG. 7. Specific activities of lipids from mitochondrial-rich fraction expressed as per cent counts per minute at one day after injection. These values are corrected for dilution.

phosphatide in young animals was metabolized somewhat faster than sphingomyelin, ethanolamine phosphatide, cholesterol, and cerebroside. Acetate-1-C^{14} injected into adult animals, however, was not well incorporated into serine phosphatide. It is suggested that the metabolic role of this lipid in myelin may change with the development of the animal.

The most serious inconsistency between our data and that of Davison is in the relative rate of turnover of choline phosphatide, a major myelin component. The difference may best be explained by postulating a more rapid turnover for the fatty acids of this phospholipid than for the glycerol and phosphorylcholine. With this possibility in mind we have begun a series of experiments in which the choline phosphatide from myelin at each age is degraded in steps, first removing the β fatty acid by phospholipase A, then hydrolyzing the lysolecithin into its fatty acid and water-soluble components. Specific activities of the three parts are determined separately. Preliminary data indicate that the β fatty acid is initially more highly labeled than the α but subsequently does not lose its activity more rapidly. At present our preliminary evidence indicates that both fatty acid chains participate in the high rate of choline phospholipid metabolism in myelin.

The fatty acids of choline phosphatide have been found to be somewhat shorter than those generally present in brain sphingolipids (21). In the Vandenheuvel model of myelin the shorter fatty acid chains do not interdigitate as do the longer chains (22; for discussion see 23). This may render these fatty acid structures more susceptible to certain kinds of enzyme action than other myelin components, even though little enzymic activity has been found in the tightly wound, highly interacting myelin structure (24).

In general, the pattern of metabolism of the lipid components of myelin, though having a different time scale, is similar to the pattern for the mitochondrial-rich fraction. Inositol phosphatide, choline phosphatide, and serine phosphatide in that order are metabolized most rapidly and in both, cholesterol and cerebroside are the slower components. Except for cerebroside, a minor fraction, cholesterol turned over much more slowly than other lipids in the mitochondrial-rich fraction. This may indicate a purely structural role for cholesterol in the membrane. The similarity of the lipid turnover pattern in the two types of membrane preparations may be indicative that this pattern is typical of nervous tissue membranes in general.

The foregoing findings and discussion have led us to the tentative conclusion that differences in metabolic rates are seen within the myelin membrane. Myelin is not entirely a metabolic unit, but certain structures are more actively metabolized than the membrane as a whole. This may suggest also that inositol phosphatide, choline phosphatide, and perhaps serine phosphatide may play a role different from other myelin lipids and that their function may not be solely structural.

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Investigation of the Biogenetic Reaction Sequence of Cholesterol in Rat Tissues, Through Inhibition with AY-9944

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Abstract

An inhibitor of Δ^7-reductase, AY-9944 (trans-1,4-bis(2-dichlorobenzylaminomethyl cyclohexane dihydrochloride), was used to investigate the last steps of cholesterol formation in brain and liver of adult and newborn rats. The accumulation of different sterols in the two tissues of the same animals was observed. $\Delta^{5,7}$-Cholestadien-3β-ol, $\Delta^{7,24}$-cholestadien-3β-ol and $\Delta^{5,7,24}$-cholestatrien-3β-ol, which are not present in detectable amounts in control brains, were identified in brains of growing rats treated with AY-9944. An accumulation of $\Delta^{5,7}$-cholestadien-3β-ol only was found in adult rat tissues.

These differences in sterol accumulation are discussed in relation with the possible in vivo pathways of cholesterol biosynthesis.

Introduction

MANY STEROLS, either found in mammalian tissues or shown to be incorporated into cholesterol, are considered as possible intermediates in the conversion of lanosterol to cholesterol (1–9). Avigan and Steinberg (10) suggested two possible series of sterol precursors of cholesterol, one with a double bond in the side chain between the carbon atoms in position 24 and 25 and the other with a saturated side chain. Uncertainty still exists on the predominant physiological sequence leading to cholesterol. The reduction of Δ^{24}, an obligate step in cholesterol formation, is probably irreversible (11) and it is independent of structure of the the steroid nucleus (11,12); it seems to occur predominantly, at least in adult rat liver, at the stage of C 28 sterols (13).

In recent years pharmacological means have been used to investigate this problem. Drugs able to block selectively the Δ^{24}-reductase, such as Triparanol (14), 20,25-diazacholesterol (15,16), 22,25-diazocholestanol (17,18), induce in tissues an accumulation of desmosterol (5,24-cholestadien-3β-ol), the last possible cholesterol precursor with a double bond in the side chain.

A specific inhibitor of the 7-dehydrocholesterol-Δ^7-reductase has also been described. This compound, trans 1-4-bis (2-dichlorobenzylaminomethyl) cyclohexane dihydrochloride (AY-9944), was shown to inhibit this enzyme at low concentrations (19,20) and to be 100 times more active than Triparanol in this respect (21). In agreement with these data, 7-dehydrocholesterol was detected in plasma and liver (19, 22) of rats treated with AY-9944. The simultaneous administration of Triparanol and AY-9944 induces, in pig liver, an accumulation of 5,7,24-cholestatrien-3β-ol (23), a sterol already considered as a possible precursor of cholesterol (7,24).

It is interesting to observe that different cholesterol precursors are present in different mammalian tissues: 7-dehydrocholesterol in the small intestine of guinea pig (9), methostenol in several tissues of rat (1), desmosterol in brain of growing rat (25,26) and in fetal brain of guinea pig and man (26). A large number of sterol precursors of cholesterol were also found in skin of normal adult rat (27).

Such observations suggest tissue-dependent differences in the activity of enzymatic systems involved in the conversion of lanosterol to cholesterol; these findings also indicate that the use of specific enzyme inhibitors may be helpful in elucidating the physiological sequence leading to cholesterol, particularly when the effects on different tissues are compared.

In our experiments an attempt has been made to obtain evidence for possible differences in sterol composition of tissues in growing and mature rats after treatment with AY-9944.

TABLE I

Steroid Number Contributions for Functional Groups on Nonselective (SE-30) and Selevtive Phase (CNSi)

Functional groups	Steroid parent	SE-30		CNSi
		a	b	c
A/B cis	Coprostane/cholestane	—0.2	—0.2	.....
A/B cis	Coprostanol/cholestanol	—0.3	—0.4	—0.55
3 β-ol (eq.)	Cholestanol/cholestane	2.4	2.4	5.90
Δ^5	Cholesterol/cholestanol	—0.1	—0.1	0
Δ^7	Lathosterol/cholestanol	0.4	0.4	0.60
$\Delta^{5,7}$	7-Dehydrocholesterol/ cholestanol	....	0.3	0.95
Δ^8	Zymosterol/ cholestanol + Δ^{24}	....	0.7	0.70
Δ^{24}	Desmosterol/cholesterol	0.3	0.3	0.70
4,4',14-methyl	Lanosterol/zymosterol	....	0.8	0.30

[a] Vandenheuvel, W. J. A., and E. C. Horning, Biochim. Biophys. Acta *64*, 416, 1962.
[b] Our data—conditions: 2 m × 3 mm glass spiral tube, 1% SE-30 on 100–140 mesh Gas-Chrom P; 211C; N_2 1.7 kg/cm²; cholestane time 18.1 min.
[c] Our data—conditions: 4 m × 3 mm glass spiral tube, 1% CNSi on 100–120 mesh Gas-Chrom P; 213C; 1.8 kg/cm²; cholestane time 7.0 min.

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